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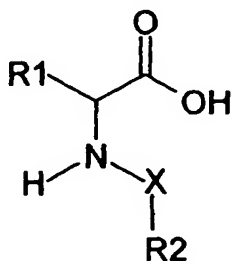
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(54) Title: FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR- $\gamma$  AGONISTS



(I)

(57) Abstract: The present invention relates to a method for treating or prevent-  
ing a PPAR- $\gamma$  mediated disease or condition comprising administration of a ther-  
apeutically effective amount of FMOC-L-Leucine or derivatives thereof, of the  
formula I. Said method is particularly useful for treating or preventing anorexia,  
hyperlipidemia, insulin resistance, inflammatory diseases, cancer and skin dis-  
orders.

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## FMOC-L-Leucine and derivatives thereof as PPAR $\gamma$ agonists

- 5 The present invention relates to a method for treating or preventing a PPAR- $\gamma$  mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine (N-(9-fluoroenylmethyloxycarbonyl)-L-Leucine) or derivatives thereof.
- 10 The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors which bind DNA as heterodimers with the retinoid X receptor (RXR) and activate a number of target genes, mainly involved in the control of lipid metabolism. PPARs have pleiotropic biological activities and wide-ranging medical applications, ranging from uses in metabolic disorders to eventual applications in inflammation, and
- 15 cancer (Desvergne and Wahli, 1999; Schoonjans *et al.*, 1997; Spiegelman and Flier, 1996). Especially, PPAR $\gamma$  has received a lot of attention because PPAR $\gamma$ -activating drugs represent a novel opportunity to treat type 2 diabetes. PPAR $\gamma$  can be activated by naturally occurring ligands, such as the long-chain fatty acid-derivatives, 15-deoxy- $\Delta$ 12,14-prostaglandin J2,  $\Delta$ 12-prostaglandin J2 (PG J2), and 9- and 13-cis-
- 20 hydroxyoctadecadienoic acid (HODE) (Forman *et al.*, 1995; Kliewer *et al.*, 1995; Nagy *et al.*, 1998). Most interesting is, however, the observation that the anti-diabetic activity of a group of the glitazones, which all possess a thiazolidinedione ring (Figure 1, Panel A), results from their PPAR $\gamma$  activating properties (Berger *et al.*, 1996; Willson *et al.*, 1996). The therapeutic efficacy of the current thiazolidinediones (TZDs)
- 25 in type 2 diabetes is, however, far from optimal and several undesirable side-effects have been reported for this drug class (Schoonjans and Auwerx, 2000). As a result, there is a need for non-TZD-based alternative ligands of PPAR $\gamma$ .

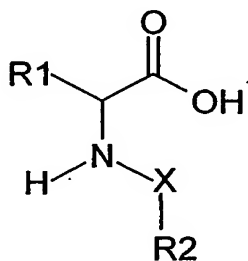
Recently, a series of L-tyrosine based PPAR $\gamma$  ligands were designed by replacing the thiazolidinedione ring with a carboxylic acid and by introducing an amine function on the adjacent carbon while keeping the parahydroxybenzyl sequence (Figure 1, Design 1 route). An optimal PPAR $\gamma$  activity was obtained when the amine on the alpha carbon of the L-tyrosine ligands was substituted with a benzoylphenyl function, leading to the development of *N*-(2-benzoylphenyl)-L-tyrosine derivatives (Figure 1, Panel B, left part) (Cobb *et al.*, 1998; Collins *et al.*, 1998). Rigidifying the benzoyl and phenyl moieties of this alpha-amino substituent through an additional phenyl-phenyl bond (Figure 1, Panel B, right part) leads to compounds with good potencies (Cobb *et al.*, 1998; Collins *et al.*, 1998).

In connection with the present invention, it was unexpectedly found that FMOC-L-tyrosine derivatives were devoid of PPAR $\gamma$  activity, whereas FMOC-L-leucine (hereafter also designated as F-L-Leu), whose structure is lacking the parahydroxybenzyl sequence present in both the TZDs and previously developed L-tyrosine-based PPAR $\gamma$  ligands (Figure 1), is a new potent insulin-sensitizing compound with unique PPAR $\gamma$ -activating and -binding properties.

F-L-Leu, referred to as NPC 15199, has been described as a drug active in various inflammatory models through an unknown anti-inflammatory mechanism (Miller *et al.*, 1993) (Burch *et al.*, 1991). But, the present invention provides new applications of this compound and derivatives thereof as a PPAR $\gamma$  agonist.

## Description

The present invention relates to a method for treating or preventing a PPAR- $\gamma$  mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:



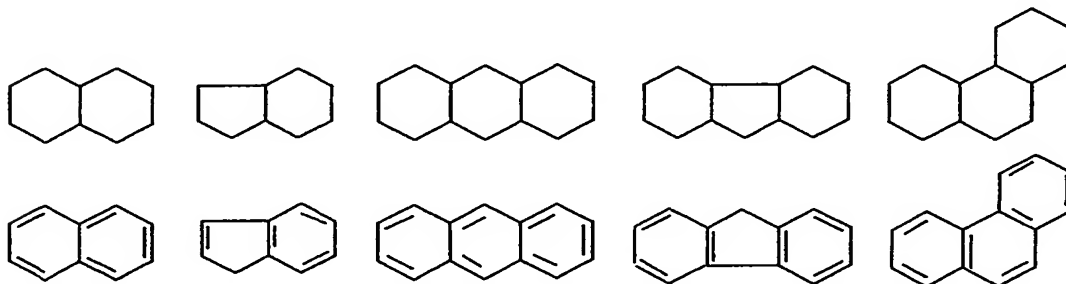
wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

10 X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,

R2 is a condensed polycyclic group comprising at least two cycles.

In a first embodiment, the R2 group comprises at least two cycles selected from carbocycles and heterocycles.

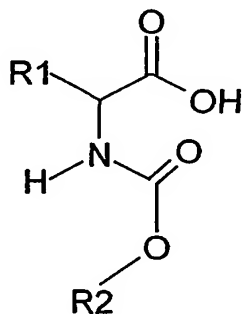
The R2 group can be advantageously selected from



wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

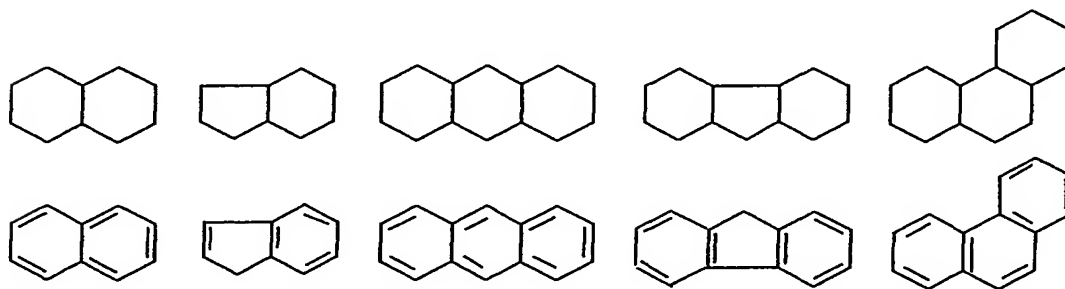
In a second embodiment, the X chain comprises one or two carbon atoms which may  
5 be substituted by an oxo group.

A preferred embodiment of the invention is directed to a method for treating or preventing a PPAR- $\gamma$  mediated disease or condition comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound  
10 is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

R2 is a polycyclic group selected from

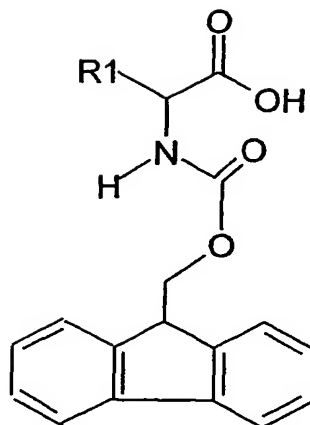


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wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

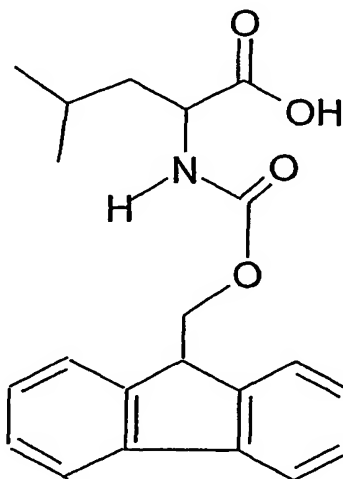
Compounds that are more particularly suitable have the formula :

5



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group  
 5 comprising from 1 to 6 carbon atoms and wherein the said tricyclic group optionally  
 comprises one to four heteroatoms selected from halogens, N, O and S.

For example, a preferred compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected  
 10 from halogens, N, O and S; such as N-(9-fluorenylmethoxycarbonyl)-L-Leucine.

The method according to the invention is useful for treating or preventing anorexia, for increasing or decreasing body weight, treating or preventing hyperlipidemia, for increasing insulin sensitivity and for treating or preventing insulin resistance, as occurs in diabetes.

5

Among the other diseases or conditions that can be treated or prevented with the compounds described above, chronic inflammatory disorders such as inflammatory bowel disease, ulcerative colitis, Crohn's disease, arthritis, notably rheumatoid arthritis, polyarthritis and asthma are relevant.

10

The invention can also be reduced to practice for cancer, notably colon, prostate and hematological cancer, as well as for atherosclerosis and skin disorders, notably psoriasis.

15 We tested a number of the FMOC-aminoacid series for PPAR $\gamma$ -binding and activation. Interestingly, whereas FMOC-L-tyrosine, which was structurally most similar to the L-tyrosine based PPAR $\gamma$  ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), was devoid of PPAR $\gamma$ -activating properties, another member of the FMOC-aminoacid series, F-L-Leu, bound and activated PPAR $\gamma$  in a comprehensive set of *in vitro* and *in vivo* tests.  
20 Evidence supporting FMOC-L-leucine as a stereoselective PPAR $\gamma$  agonist ligand is provided by the following arguments:

- 1) F-L-Leu binds *in vitro* to PPAR $\gamma$  as evidenced by ESI-mass spectrometry (figure 5 and protease protection assays (figure 4);
- 2) F-L-Leu enhanced co-activators recruitment to the PPAR $\gamma$  protein (figure 6);
- 25 3) F-L-Leu activates PPAR $\gamma$  in cotransfection studies (figure 3);
- 4) F-L-Leu induces adipocyte differentiation as judged by increased lipid accumulation and the induction of adipocyte target genes, such as LPL and aP2 (figure 7);

- 5) F-L-Leu acts as a potent insulin-sensitizing agent in both diabetic and more interestingly also in non-diabetic murine models (figure 8);
- 6) Finally, like the TZDs ((Jiang *et al.*, 1998; Su *et al.*, 1999), F-L-Leu also has significant anti-inflammatory activities and could prevent inflammatory bowel disease (figure 9). Since F-L-Leu is clearly structurally different from thiazolidinediones and L-tyrosine based PPAR $\gamma$  ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998) and since F-L-Leu presents little or no structural analogies with the partial agonists GW0072 (Oberfield *et al.*, 1999) and L-764406 (Elbrecht *et al.*, 1999) and the antagonist BADGE (bisphenol A diglycidyl ether) (Wright *et al.*, 2000), F-L-Leu defines a chemically new class of PPAR $\gamma$  ligands.

Although F-L-Leu shares several functional characteristics with known PPAR $\gamma$  ligands, an important number of features distinguish F-L-Leu from these compounds which will be addressed hereinafter.

15

F-L-Leu possesses an acidic function with the ability to liberate a proton, provided by its carboxylic group. This is a feature shared by the natural ligand, PG J2, as well as previously developed L-tyrosine based ligands. Such an acidic function is also present in the TZD ring at the level of the nitrogen located between the two carbonyl groups. A carboxylic group is also recovered in other PPAR $\gamma$  ligands such as GW0072, a weak partial agonist which antagonizes adipocyte differentiation, but in which lateral side chain substitution is approximately ten carbon atoms distant from the carboxylate (Oberfield *et al.*, 1999). This distance is in contrast with agonists such as the tyrosine derived ligands and F-L-Leu where a side-chain substitution occurs on the alpha-amino position. The stereoselectivity of the activation of PPAR $\gamma$  with the FMOC L- and D leucines (L- by far more potent than the D-enantiomer) confirms previous observation

25



made on other ligands with an asymmetric carbon including 8-HETE (S>R) and an alpha-trifluoroethoxy-propanoic acid derivative (S>R) (Rangwala *et al.*, 1997; Young *et al.*, 1998; Yu *et al.*, 1995).

5 Experimental evidence suggests that F-L-Leu would interact with the ligand binding site of PPAR $\gamma$  in a fashion distinct from both the TZDs and the tyrosine-based ligands. Nuclear receptors generally only will dock one ligand molecule in their ligand binding pocket, which fits rather tightly around the ligand. Binding of both TZDs and tyrosine-based PPAR $\gamma$  ligands follows this paradigm of "1 ligand/1 receptor" (Willson *et al.*  
10 2000). The rather spacious ligand binding pocket of PPAR $\gamma$ , however, would not only allow the binding of large ligands, such as the tyrosine-based ligands, but eventually also allow binding of multiple ligand molecules to a single receptor. Our ESI-mass spectrometry data confirm that this is in fact the case with the F-L-Leu, where two molecules are shown to be bound to PPAR $\gamma$  ligand binding domain.

15

The capacity of PPAR $\gamma$  to bind two molecules of F-L-Leu could underly in fact some of the particular biological characteristics of this ligand. In fact, in transfection experiments F-L-Leu was two orders of magnitude less potent than the TZD rosiglitazone, although both compounds had similar maximal efficacy. The low  
20 potency and the steep dose-response curve could therefore be explained by the fact that a higher molar ratio of ligand to receptor is required to change its configuration, a finding consistent both with the results of our protease protection (figure 4) and cofactor interaction assays (figure 6).

25 Although, less potent *in vitro*, F-L-Leu compares rather favorably to TZDs, such as rosiglitazone, for anti-diabetic activity *in vivo*. Administration of F-L-Leu (10

mg/kg/day) to the diabetic db/db mice improved insulin sensitivity more dramatically than an equivalent dose of rosiglitazone. This could be deduced from the more robust reduction of the AUC in IPGTT for an almost equivalent reduction in fasting insulin levels. Furthermore, at a dose of 30 mg/kg/day F-L-Leu was able to significantly

5 improve insulin sensitivity in normal animals, an effect never observed with glitazones. One caveat with this comparison between the *in vivo* efficacy of F-L-Leu relative to the TZDs, lies in the intraperitoneal route of drug administration used here. This is the only route described at present to be effective for F-L-Leu, but it is known to be suboptimal for TZDs, which are readily orally bioavailable. Despite this potential

10 draw-back, the results obtained with F-L-Leu as a potential anti-diabetic drug remain, however, remarkable. Moreover, F-L-Leu structure does not share a TZD ring, but offers a isosteric version of this chemical group via a carboxylic function (see figure 1), which is devoid of TZD-related side effects.

15 In summary, we describe F-L-Leu as a small synthetic PPAR $\gamma$  ligand. Unlike known PPAR $\gamma$  ligands, two molecules of F-L-Leu bind to a single PPAR $\gamma$  molecule, making its mode of receptor interaction novel and interesting. This unique way of receptor interaction, underlies some of the particular pharmacological properties of F-L-Leu. In general, F-L-Leu exerts similar biological activities as the known groups of PPAR

20 agonists, with a distinct pharmacology, characterized by a lower potency, but similar maximal efficacy. This novel synthetic molecule represents hence a new pharmacophore, which can be optimized according to routine procedures, for modulation of PPAR $\gamma$  biological activity.

### Figure legends

**Figure 1: Schematic representation of PPAR $\gamma$  ligand structures.** The different routes followed for the design are indicated.

- 5 A. anti-diabetic glitazones
- B. L-tyrosine based PPAR $\gamma$  ligands
- C. FMOC amino acids

**Figure 2: Modulation of transcriptional activity of PPAR $\gamma$ 2 by FMOC-amino-acids in Hep G2 cells.** Hep G2 cells were co-transfected with an expression vector for PPAR $\gamma$ 2 (0.1  $\mu$ g/well), pGL3-(J<sub>wt</sub>)<sub>3</sub>TKLuc reporter construct (0.5  $\mu$ g/well), and pCMV- $\beta$ Gal (0.5  $\mu$ g/well), as a control of transfection efficiency (0.5  $\mu$ g/well). They were then grown during 24 h in the presence or absence of indicated compound. Activation is expressed as relative luciferase activity/ $\beta$ -galactosidase activity. Each point was performed in triplicate. This figure is representative of three independent experiments.

**Figure 3: F-L-Leu enhances transcriptional activity of PPAR $\gamma$ 2 in different cell lines.** RK13 cells (A and D), CV1 cells (B) or Hep G2 cells (C) were co-transfected with an expression vector for PPAR $\gamma$ 2 (0.1  $\mu$ g/well), pGL3-(J<sub>wt</sub>)<sub>3</sub>TKLuc reporter construct (0.5  $\mu$ g/well), and pCMV- $\beta$ Gal (0.5  $\mu$ g/well), as a control of transfection efficiency (0.5  $\mu$ g/well). They were then grown during 24 h in the presence or absence of indicated compound. Activation is expressed as relative luciferase activity/ $\beta$ -galactosidase activity. Each point was performed in triplicate, and each figure is representative of four independent experiments.

**Figure 4: F-L-Leu ligand alters the conformation of PPAR $\gamma$ .** <sup>35</sup>S-PPAR $\gamma$  was synthesized *in vitro* in a coupled transcription/translation system. Labeled PPAR $\gamma$  was subsequently incubated with DMSO (0.1%), rosiglitazone (10<sup>-4</sup>M) or F-L-Leu (10<sup>-4</sup>M).

followed by incubation with distilled water or increasing concentrations of trypsin. Digestion products were analyzed by SDS-PAGE followed by autoradiography. The migration of intact PPAR $\gamma$  is indicated and the asterisk indicates the 25-kDa resistant fragment of PPAR $\gamma$ .

5

**Figure 5: Two molecules of F-L-Leu bind to a single PPAR $\gamma$  molecule.** ESI-mass spectrometry analysis.

**Figure 6: F-L-Leu enhances the interaction of PPAR $\gamma$  with p300.** The purified his-tagPPAR $\gamma$ <sub>2DE203-477</sub> protein was incubated with purified p300Nt-GST protein and glutathione-Q-Sepharose beads in presence of DMSO (0.1%), rosiglitazone ( $10^{-4}$  M) or F-L-Leu ( $10^{-3}$  M). The beads were then washed and the samples separated on SDS-PAGE and blotted. The blot was developed with anti-histidine antibodies.

**Figure 7: F-L-Leu enhances adipocyte differentiation.** Confluent 3T3-L1 cells were incubated with 2  $\mu$ M insulin, 1  $\mu$ M dexamethasone, and 0.25 mM isobutyl methyl xanthine for two days. Then, the cells were incubated in presence of DMSO (0.1 %), F-L-Leu ( $10^{-5}$  M) or rosiglitazone ( $10^{-7}$  M) for 4 days. **A:** RNA was isolated from 3T3-L1 cells after different times of differentiation induction. Blots were hybridized with 36B4 (to control for RNA loading); LPL or aP2 cDNAs. **B:** Cells were stained with Oil Red O after 6 days. LPL: lipoprotein lipase.

**Figure 8: F-L-Leu improves insulin sensitivity in C57BL/6j and db/db mice.** Intraperitoneal glucose tolerance test (IPGTT) in C57BL/6j (**A**) or db/db (**B**) mice 10 to 12 weeks old (n=8). Diamonds correspond to DMSO-treated mice; squares to F-L-Leu-treated mice at the concentration of 10 mg/kg/day and triangles to F-L-Leu-treated mice at the concentration of 30 mg/kg/day (for C57BL/6j mice, **A**) or rosiglitazone-treated mice at the concentration of 10 mg/kg/day (for db/db mice, **B**). Insulinemia (**C**) and body weights (**D**) of db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day).

**Figure 9: F-L-Leu protects against colon inflammation in TNBS-treated Balb/c mice. A: Ameho histologic scores (left panel) and survival rate (right panel) in TNBS-treated mice injected either with DMSO or F-L-Leu (50 mg/kg/day). B: TNF $\alpha$  and IL-1 $\beta$  mRNA levels in the colon of TNBS-treated mice injected with DMSO or F-L-Leu (50 mg/kg/day). Results are expressed as mean  $\pm$  SEM.**

The following materials and methods were used to perform the examples below.

## 10 **Materials and methods**

FMOC-derivatives were acquired at Sen Chemicals (Dielsdorf, Switzerland). Rosiglitazone and pioglitazone were kind gifts of Dr. R. Heyman (Ligand Pharmaceuticals, San Diego, CA). The antibodies directed against the AB domain of PPAR $\gamma$  were produced in our laboratory (Fajas *et al.*, 1997). The protease inhibitor cocktail was purchased at ICN (Orsay, France).

### **Cell culture and transient transfection assays**

The CV1, RK-13, and Hep G2 cell lines were obtained from ATCC (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics. Transfections with chloramphenicol acetyltransferase (CAT) or luciferase (luc.) reporter constructs were carried out exactly as described previously (Schoonjans *et al.*, 1996). The pGL3-(J<sub>wt</sub>)<sub>3</sub>TKLuc and the pGL3-(J<sub>wt</sub>)<sub>3</sub>TKCAT reporter constructs contain both three tandem repeats of the J site of the apolipoprotein A-II promoter cloned upstream of the herpes simplex virus thymidine kinase (TK) promoter and the luciferase or the CAT reporter genes respectively (Vu-Dac *et al.*, 1995). The following

expression vectors were used; pSG5-hPPAR $\gamma$ 2, a construct containing the entire cDNA of the human PPAR $\gamma$ 2 (hPPAR $\gamma$ 2) (Fajas *et al.*, 1997); pSG5-mPPAR $\alpha$  (Isseman *et al.*, 1993); and pCMV- $\beta$ Gal, as a control of transfection efficiency.

## 5     **Production of proteins and mass spectrometry**

The p300Nt-GST, fusion protein was generated by cloning the N-terminal part of the p300 protein (a.a. 2 to 516) downstream of the glutathione-S-transferase (GST) protein in the pGex-T1 vector (Pharmacia, Orsay, France). The fusion proteins were then expressed in *Escherichia coli* and purified on a glutathione affinity matrix (Pharmacia). Human PPAR $\gamma$  (aa. 203 to 477 of PPAR $\gamma$ ) was subcloned into the  
10     pET15b (Novagen, Madison, WI) expression vector. The his-tagPPAR $\gamma$ 2<sub>DE203-477</sub> proteins were produced as follow. The protein was purified using a metal chelate affinity column with an affinity column Co<sup>2+</sup> coupled agarose (High Trap chelatin, Pharmacia). The protein was eluted with 20mM Tris-HCl, 500mM NaCl, 130mM  
15     imidazole and 1-2 propanediol 2.5% (pH 8.5). A second purification step was made by gel filtration (Superdex 200 16/60, Pharmacia). The protein was eluted with 20mM Tris-HCl, 100mM NaCl, 5mM DTT and 1-2 propanediol 2,5% (pH 8.5).  
Liquid chromatography-electrospray ionization (ESI)-mass spectrometry analysis was performed as previously described (Rogniaux *et al.*, 1999).

20

## **Protease protection and pull-down experiments**

*Protease protection experiments.* The pSG5-hPPAR $\gamma$ 2 plasmid was used to synthesize <sup>35</sup>S-radiolabeled PPAR $\gamma$  in a coupled transcription/translation system according to the protocol of the manufacturer (Promega, Madison, WI). The transcription/translation  
25     reactions were subsequently aliquoted into 22.5  $\mu$ l and 2,5  $\mu$ l of phosphate buffered saline +/- compound were added. The mixture was separated into 4.5  $\mu$ l aliquots and 0.5  $\mu$ l of distilled water or distilled water-solubilized trypsin were added. The protease

digestion were allowed to proceed for 10 min at 25°C and terminated by the addition of denaturing loading buffer. After separation of the digestion products in a gel SDS PAGE 12% acrylamide, the gel was fixed in 10% acetic acid (v/v): 30% ethanol (v/v) for 30 min, treated in Amplify™ (Amersham, Orsay, France) and dried. The radiolabeled digestion products were visualized by autoradiography.

*Pull-down experiments.* The purified his-tagPPAR $\gamma$  DE proteins were incubated 1 hour at 22°C in pull-down buffer (phosphate-buffered saline 1x, Glycerol 10%, NP40 0,5% with either GST or p300Nt-GST fusion protein, glutathione-Q sepharose beads, and F L-Leu ( $10^{-3}$ M) or rosiglitazone ( $10^{-4}$ M) when necessary. The beads were then washed 4 times in pull-down buffer and boiled in 2x sample buffer. The samples were separated by 12% acrylamide SDS-PAGE and transferred to nitrocellulose membrane. Blots were developed with antibodies directed against polyhistidine amino acid sequences.

### 15 Adipocyte differentiation

3T3-L1 cells (ATCC, Rockville, MD) were grown to confluence in medium A (Dulbecco's modified Eagle's Medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 $\mu$ g/ml streptomycin). Confluent cells were incubated in medium A containing 2  $\mu$ M insulin, 1  $\mu$ M dexamethasone, and 0.25 mM isobutyl methyl xanthine for two days. Then, the cells were incubated in medium A in presence or absence of PPAR $\gamma$  agonist for 4 days, changing the medium every 2 days. Adipogenesis was evaluated by analysis of the expression of adipocyte-specific markers and by staining of lipids with Oil Red O (Chawla and Lazar, 1994).

### RNA preparation and analysis

RNA was isolated from 3T3-L1 cells by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis of total cellular RNA was performed as described (Auwerx *et al.*, 1989)

5 Lipoprotein lipase (LPL), aP2 and 36B4 were used as probes (Graves *et al.*, 1992 Laborda, 1991; Lefebvre *et al.*, 1997). For RT-competitive PCR, total RNA (5-10 $\mu$ g) was reverse transcribed into complementary DNA (cDNA) (Desreumaux *et al.*, 1999 Fajas *et al.*, 1997). The RT reaction mixture was amplified by PCR using sense and antisense primers specific for  $\beta$ -actin, TNF $\alpha$  and IL-1 $\beta$ . The samples were subjected to

10 40 PCR cycles, consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 52-58°C, and primer extension for 1.5 min at 72°C using a Gene Amp PCR System 9700 (Perkin-Elmer Corporation, Foster City, CA). The quantity of mRNA was expressed as the number of TNF $\alpha$  or IL-1 $\beta$  cDNA per  $\beta$ -actin cDNA molecules.

### 15 Animal experiments, glucose metabolism and inflammation

All mice were maintained in a temperature-controlled (25 °C) facility with a strict 12 h light/dark cycle and were given free access to food (standard mice chow; DO4, UAR France) and water. Animals received F-L-Leu or rosiglitazone by intraperitoneal injection.

20 C57Bl/6J and db/db mice (8 per group) were obtained through the Janvier laboratories (Laval-Le Genest, France). Intraperitoneal glucose tolerance tests (IPGTT) were performed as described (Kaku *et al.*, 1988). Briefly, mice were fasted overnight (18 h) and injected intraperitoneally (i.p.) with 25 % glucose in sterile saline (0.9 % NaCl) at a dose of 2 g glucose/kg body weight. Blood was subsequently collected from the tail

25 for glucose quantification with the Maxi Kit Glucometer 4 (Bayer Diagnostic, Puteaux France) prior to and at indicated times after injection. Blood for insulin measurement



was collected in fasting mice from the retroorbital sinus plexus under chloroform anesthesia. Plasma was separated and insulin measured using a radio immunoassay kit (Cis bio international, Gif-sur-Yvette, France).

Male Balb/c mice (8 per group) were used for the colitis studies (Jackson laboratories, Bar Harbor, Maine). Colitis was induced by administration of 40  $\mu$ l of a solution of TNBS (150 mg/kg, Fluka, Saint Quentin Fallavier, France) dissolved in NaCl 0.9% and mixed with an equal volume of ethanol (50% ethanol). This solution was administered intrarectally via a 3.5 F catheter (Ref EO 3416-1, Biotrol, Chelles, France) inserted 4 cm proximal to the anus in anesthetized mice [Xylazine (50 mg/kg of Rompun® 2%, Bayer Pharma, Puteaux, France) and Ketamine (50 mg/kg of Imalgene® 1000, Rhône Mérieux, France)]. Animals were sacrificed by cervical dislocation under ether anesthesia two days after TNBS administration. The colon was quickly removed, opened, washed. A 2 cm colonic specimen located precisely 2 cm above the anal canal was dissected systematically in 4 parts. One part was fixed overnight in 4% paraformaldehyde acid at 4°C, dehydrated in alcohol and embedded in paraffin. Sections (5  $\mu$ m) were then deparaffined with xylene and rehydrated by ethanol treatment. Stained sections with haematoxylin-eosin were examined blindly by a pathologist and scored according to the Ameho criteria (Ameho *et al.*, 1997). The other parts of the colon were used for RNA isolation for the quantification of TNF $\alpha$  and IL1 $\beta$  mRNA expression.

### Statistical analysis

Values were reported as mean +/- standard deviation. Statistical differences were determined by the Mann-Whitney U test. P<0.05 was accepted as statistically significant.

**Example 1 : FMOC-L-leucine activates PPAR $\gamma$  in cell transfection experiments**

Various FMOC derivatives of unsubstituted (L-tyrosine, D-leucine, and L-leucine) aminoacids were tested and compared to rosiglitazone or pioglitazone (as positive  
5 internal controls) for their ability to activate PPAR $\gamma$  in transient transfection experiments in HepG2 cells using the pSG5-hPPAR $\gamma$ 2 expression and J<sub>3</sub>TKpGl<sub>3</sub> reporter plasmids. In contrast to L-tyrosine PPAR $\gamma$  ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), the FMOC substituted L-tyrosine derivative did not activate PPAR $\gamma$ . Significant PPAR $\gamma$  activity could, however, be detected for F-L-leu at the  
10 concentration of  $10^{-5}$  M (figure 2). In contrast, no significant PPAR $\gamma$  activation was detected with the FMOC-D-leucine derivative, demonstrating that PPAR $\gamma$  activating properties of F-L-leu were stereoselective. Additional transfection experiments with F-L-Leu were performed on different cell lines (RK13, CV1 and HepG2 cells) (figure 3 A, B and C). In the rabbit kidney RK13 cells, we found that rosiglitazone has an  
15 optimal activity between  $10^{-8}$  to  $10^{-7}$  M. For F-L-Leu, PPAR $\gamma$  activation occurred at concentrations of  $10^{-5}$  M (figure 3A). Consistent with previous results, F-L-Leu concentrations of  $10^{-5}$  M were also required for optimal PPAR $\gamma$  activation in simian renal cells CV1 (figure 3B), and in human HepG2 cells (figure 3C). The optimal concentration for PPAR $\gamma$  activation by F-L-Leu was similar to that of PG J2 and 100-  
20 fold higher than the concentration of rosiglitazone (figure 3C) or pioglitazone (data not shown) necessary to reach the same efficacy.

Finally, we tested whether FMOC-amino acid derivatives synergized or antagonized rosiglitazone activation of PPAR $\gamma$  in RK13 cells (figure 3D). No significant  
25 modification of PPAR $\gamma$  activity was observed when we added either F-L-Leu, FMOC L-tyrosine or FMOC D-leucine ( $10^{-5}$  M) to a saturating concentration of rosiglitazone.

These results furthermore confirmed (see figure 3A) that we reached maximal PPAR $\gamma$  activation using rosiglitazone and F-L-Leu at the concentration of  $10^{-7}$ M and  $10^{-5}$ M respectively.

## 5     **Example 2: Fmoc L-leucine changes PPAR $\gamma$ conformation**

Thiazolidinediones can induce an alteration in the conformation of PPAR $\gamma$ , as assessed by generation of protease-resistant bands following partial trypsin digestion of recombinant receptor (Berger *et al.*, 1999; Elbrecht *et al.*, 1999). Upon incubation of rosiglitazone with PPAR $\gamma$ , a fragment of approximately 25 kDa is protected from trypsin digestion whereas no protection is detected when PPAR $\gamma$  is incubated with DMSO vehicle (figure 4). Interestingly, F-L-Leu produced a protease protection pattern similarly to rosiglitazone, demonstrating that F-L-Leu alters PPAR $\gamma$  conformation (figure 4).

15

## **Example 3: Two molecules of Fmoc-L-leucine interact with PPAR $\gamma$**

Electrospray ionization (ESI) mass spectrometry of hPPAR $\gamma$  LBD (amino acid 203 to 477) was used to identify the specific binding of F-L-Leu with PPAR $\gamma$  (figure 5). The purified fragment of PPAR $\gamma$  LBD was incubated with vehicle alone or either 1 or 20 equivalents of F-L-Leu per equivalent of PPAR $\gamma$ . The mass of the receptor was determined after incubation by ESI-mass spectrometry. At 1 equivalent of F-L-Leu per equivalent of PPAR $\gamma$ , we could distinguish three populations of PPAR $\gamma$  corresponding to: 1/ unliganded PPAR $\gamma$ ; 2/ a complex formed by 1 PPAR $\gamma$  LBD molecule and 1 F-L-Leu molecule; and 3/ a complex formed by 1 PPAR $\gamma$  LBD molecule and 2 F-L-Leu molecules. Interestingly, when we increased the F-L-Leu concentration (8 equivalents of F-L-Leu per 1 equivalent of PPAR $\gamma$ ), we detected only the complex corresponding

25

to the PPAR $\gamma$  LBD bound with 2 F-L-Leu molecules. These results indicate that two molecules of F-L-Leu interact with one molecule of the PPAR $\gamma$  in a highly specific manner.

#### 5     **Example 4: FMOC-L-leucine enhances PPAR $\gamma$ /p300 interaction**

PPAR $\gamma$  has been previously reported to interact with the cofactor p300. The overall molecular PPAR $\gamma$ /p300 interaction was the resultant of a ligand-independent binding of p300 to PPAR $\gamma$ 's ABC domain and a ligand-dependent interaction of p300 with the  
10     PPAR $\gamma$  DE domains (Gelman *et al.*, 1999). Hence the purified PPAR $\gamma$  DE protein represents a tool to study the efficacy of PPAR $\gamma$  ligand binding properties in view of its' ability to recruit p300 upon ligand binding. Compared to the DMSO control, both rosiglitazone and F-L-Leu effectively induced the formation of PPAR $\gamma$  DE/p300Nt-GST complexes. This confirms that the F-L-Leu is a PPAR $\gamma$  ligand and that its  
15     binding to the PPAR $\gamma$  DE domain is capable of inducing conformational changes required for association with p300. The potency of the F-L-Leu compound was in this assay 2- to 3-fold lower than that of rosiglitazone.

#### **Example 5: FMOC-L-leucine induces adipocyte differentiation**

20

The ability of F-L-Leu and rosiglitazone to stimulate adipocyte differentiation of murine pre-adipocyte 3T3-L1 cells were next compared. Adipogenesis was monitored by analysis of lipoprotein lipase (LPL) and aP2 mRNA levels as markers of adipocyte differentiation and by studying morphological changes associated with the  
25     differentiation process. F-L-Leu at the concentration of  $10^{-5}$  M significantly stimulated both LPL and aP2 mRNA levels to an extent close to that seen in cells incubated with

rosiglitazone at the concentration of  $10^{-7}$  M (figure 7A). Staining of 3T3-L1 cells with Oil Red O, as a marker for neutral lipid accumulation, was performed after a 6 days incubation of cells with either DMSO, or the two PPAR $\gamma$  ligands F-L-leu or rosiglitazone (Figure7B). The two drugs were again capable of inducing neutral lipid  
5 accumulation. Hence, like rosiglitazone, F-L-Leu was an adipogenic drug in 3T3-L1 cells.

**Example 6: FMOC-L-leucine improves insulin sensitivity *in vivo***

10 To assess whether F-L-Leu could improve insulin sensitivity, we compared the glucose tolerance in C57BL/6j mice treated with F-L-Leu relative to that observed in control animals which received only the vehicle, DMSO (figure 8A). Mice were treated with 2 different doses of F-L-Leu (10 and 30 mg/kg/days) during 7 days and then IPGTT was performed. Intra-peritoneally administrated glucose was cleared in a comparable rate in  
15 mice receiving vehicle or F-L-Leu at 10 mg/kg/day. In mice treated with F-L-Leu at 30 mg/kg/day, the maximum glucose levels increased only to 320 mg/dl whereas the glucose levels climbed to 440 mg/dl after glucose injection for both 10 mg/kg/day F-L-Leu and the control group. Furthermore, the area under the curve was significantly lower in mice treated with F-L-Leu at 30 mg/kg/day relative to either control mice or  
20 mice receiving F-L-Leu at lower dose.

We next compared glucose tolerance in db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day) during 7 days. In control mice (DMSO group), glycemia rapidly increased after glucose loading, reaching a maximum of 500  
25 mg/dl between 45 to 60 min after injection, before slowly decreasing. In rosiglitazone-treated mice, glucose loading was better "tolerated" than in control animals with a

reduction in the maximal glycemia (350 mg/dl), and a more rapid recovery of these supranormal values. F-L-Leu-treated animals showed the best glucose tolerance test, with a maximal glucose level (420 mg/dl) 20 min after injection and an immediate and fast subsequent decrease to normal (100 mg/dl) values within 120 min. Furthermore, 7 days treatment of animals with F-L-Leu and rosiglitazone resulted in a dose-dependent lowering of fasting serum insulin levels (mean values of 70  $\mu$ UI/mL for db/db mice treated with either F-L-Leu or rosiglitazone versus 180  $\mu$ UI/mL for the DMSO group) (figure 8C). These data clearly show that F-L-Leu improves insulin sensitivity in both diabetic and normal mice. Interestingly, whereas rosiglitazone had a tendency to increase body weight of mice, no difference in body weight was seen in mice treated with F-L-Leu during 8 days when compared to control mice (figure 8D). In addition, we observed a tendency to diminution of the liver weight for F-L-Leu-treated mice relative to control or rosiglitazone-treated mice (data not shown).

#### Example 7: FMOC-L-leucine protects against colitis

Intrarectal administration of TNBS has been shown to induce rapidly and reproducibly a colitis in mice as a result of covalent binding of TNP residues to autologous host proteins leading to a mucosal infiltration by polynuclear cells, the production of TNF $\alpha$ , and the activation of NF $\kappa$ B (Allgayer *et al.*, 1989; Stenson *et al.*, 1992; Su *et al.*, 1999). We determined the survival rate and scored the colon damage as well as the production of cytokines two days after intra-rectal TNBS administration in control animals or animals which were treated 4 days with F-L-Leu at 50 mg/kg/day (figure 9). Interestingly, 100% of F-L-Leu-treated mice survived colon inflammation whereas only 76 % of control mice were alive after induction of inflammation. Administration of F-L-Leu furthermore reduced significantly the histologic score indicating that F-L-

Leu reduces ulceration, erosion and necrosis induced by inflammation. Finally, F-L-Leu administration resulted in a significant decrease in the mRNA levels expression of the pro-inflammatory cytokines,  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  suggesting that, like with rosiglitazone,  $\text{PPAR}\gamma$  activation by F-L-Leu protects against colon inflammation by

5 inhibition of the  $\text{TNF}\alpha$  signaling pathway.

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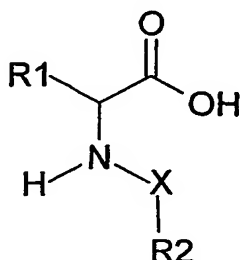
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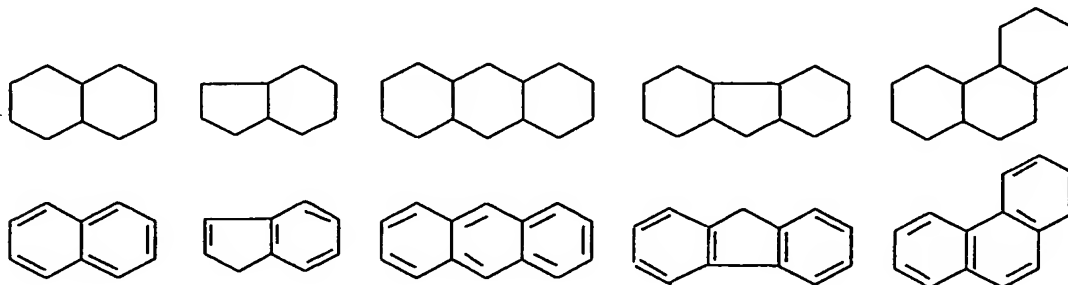
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CLAIMS

1. A method for treating or preventing a PPAR- $\gamma$  mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:

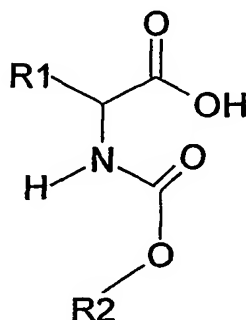


- wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,  
 X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,  
 10 R2 is a condensed polycyclic group comprising at least two cycles.
2. A method according to claim 1 wherein the R2 group comprises at least two cycles selected from carbocycles and heterocycles.
- 15 3. A method according to one of claims 1 and 2 wherein the X chain comprises one or two carbon atoms which may be substituted by an oxo group.
4. A method according to one of claims 1 to 3 wherein R2 is a polycyclic group selected from



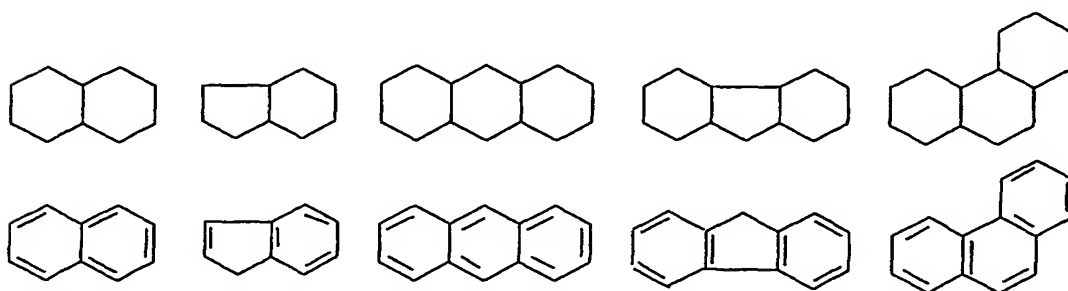
wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

- 5 5. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

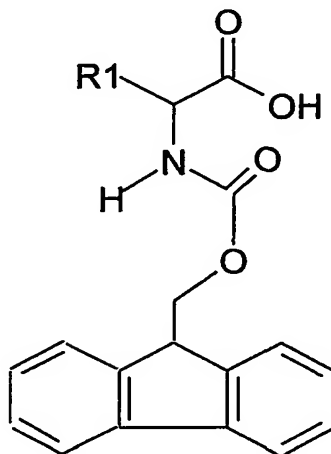
- 10 R2 is a polycyclic group selected from



wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

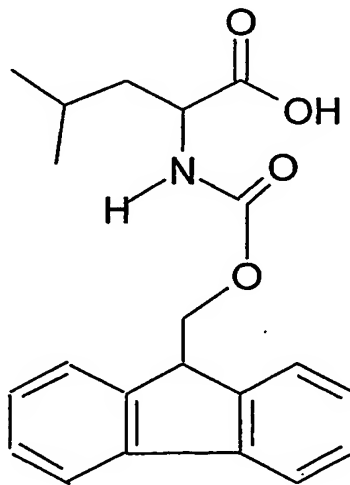


6. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group  
5 comprising from 1 to 6 carbon atoms and  
wherein the said tricyclic group optionally comprises one to four heteroatoms selected  
from halogens, N, O and S

7. A method according to claim 1 comprising administration of a therapeutically  
10 effective amount of a compound the formula I, wherein said compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S

8. A method according to claim 1 comprising administration of a therapeutically  
5 effective amount of N-(9-fluoroenylmethyloxycarbonyl)-L-Leucine.

9. A method according to one of claims 1 to 8 wherein said disease or condition is anorexia.

10 10. A method according to one of claims 1 to 8 for increasing or decreasing body weight.

11. A method according to one of claims 1 to 8 for increasing insulin sensitivity.

15 12. A method according to one of claims 1 to 8 for treating or preventing insulin resistance, as occurs in diabetes.

13. A method according to one of claims 1 to 8 wherein said disease or condition is a chronic inflammatory disorder.

20

14. A method according to one of claims 1 to 8 wherein said disease or condition is inflammatory bowel disease, ulcerative colitis or Crohn's disease.

15. A method according to one of claims 1 to 8 wherein the said disease or condition  
25 is arthritis, notably rheumatoid arthritis, polyarthritis and asthma.

16. A method according to one of claims 1 to 8 wherein said disease is cancer.

17. A method according to one of claims 1 to 8 wherein said disease is atherosclerosis.

30

18. A method according to one of claims 1 to 8 wherein said disease is a skin disorder, notably psoriasis.

19. A method according to one of claims 1 to 8 wherein said disease is  
5 hyperlipidemia.

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Figure 1C

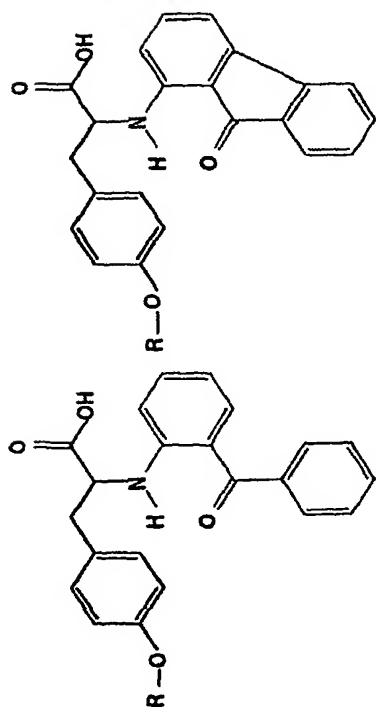
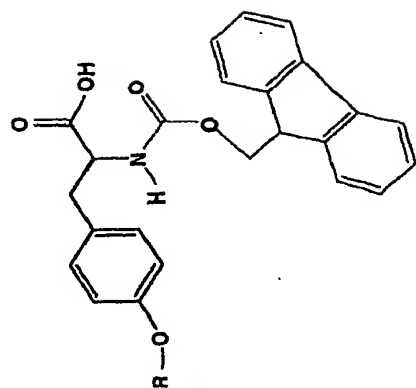


Figure 1B

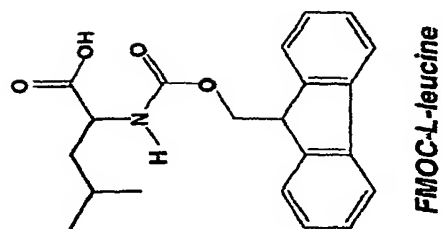
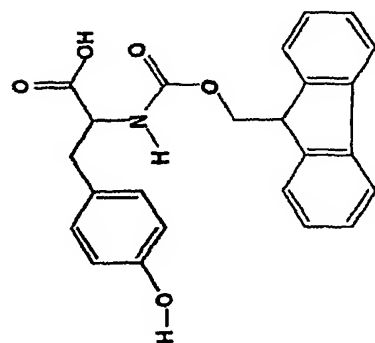
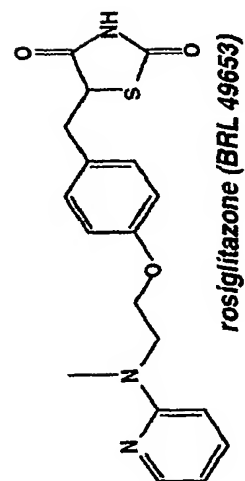
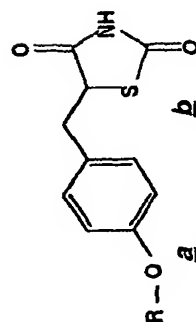
**Design1****Fmoc-L-leucine****Fmoc-L-tyrosine****rosiglitazone (BRL 49653)**

Figure 1A

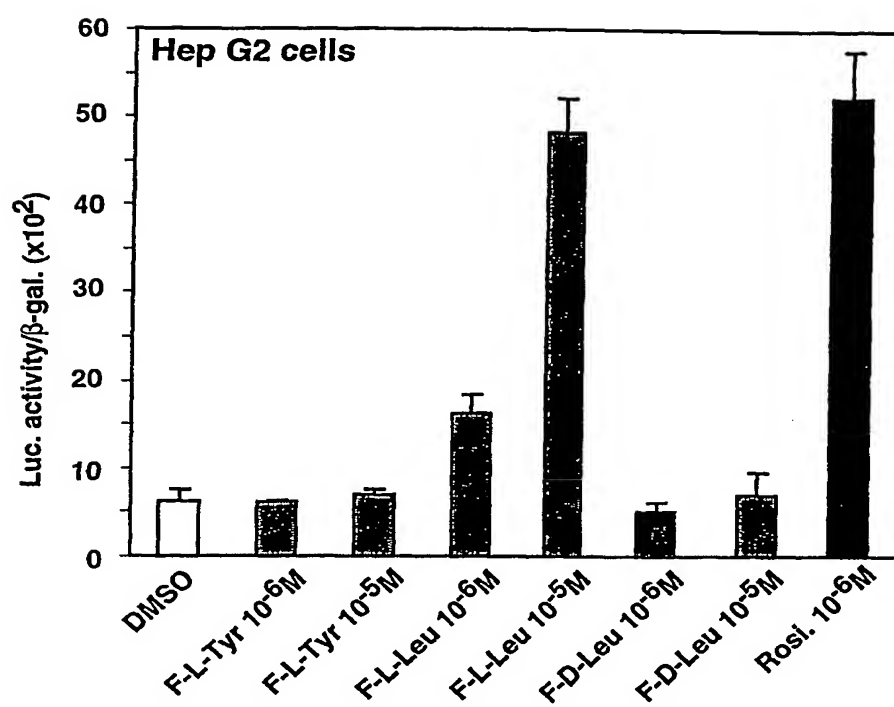
**Figure 2**

Figure 3A

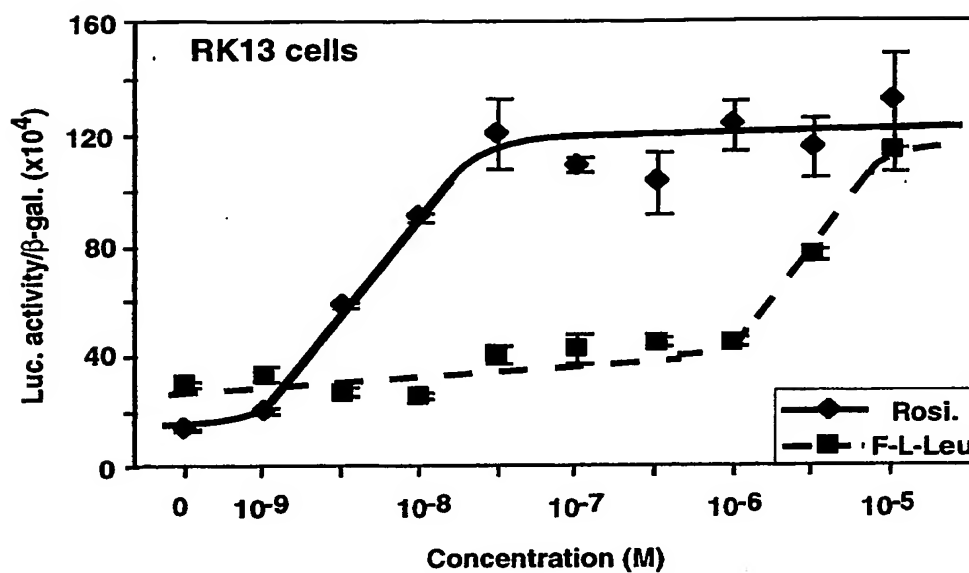
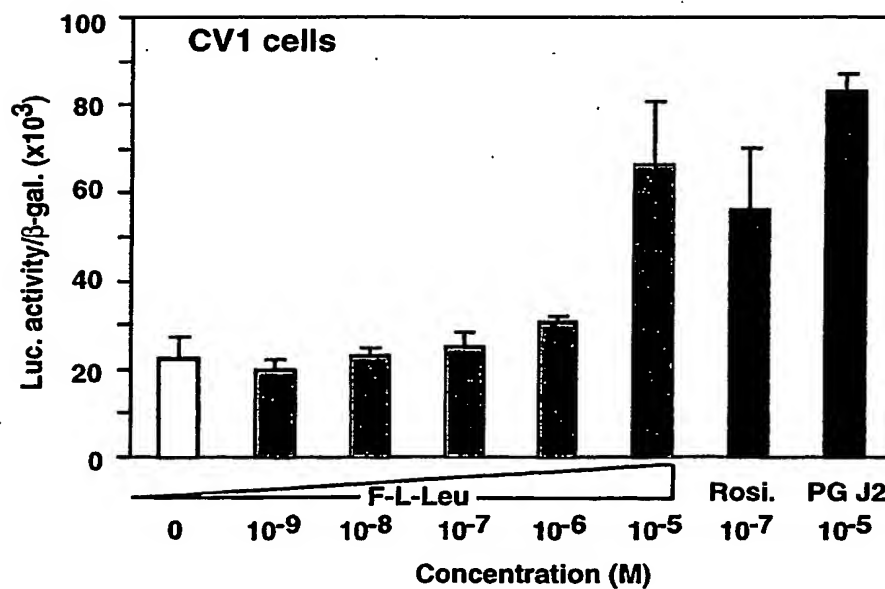


Figure 3B



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Figure 3C

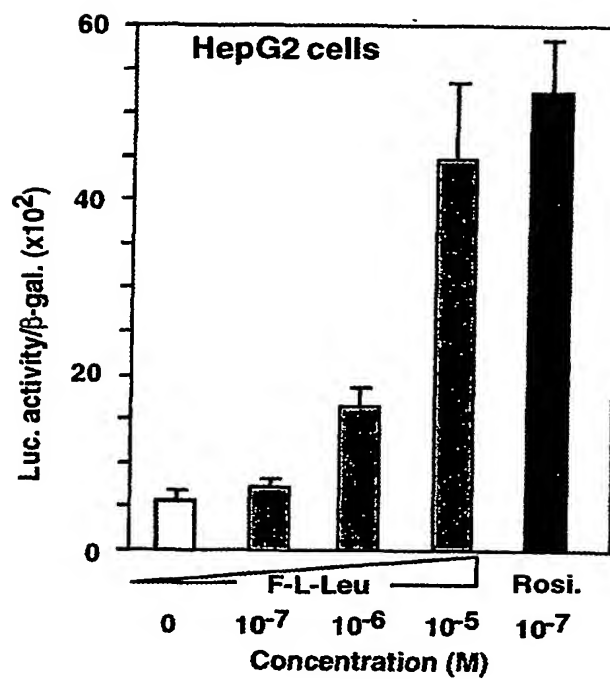
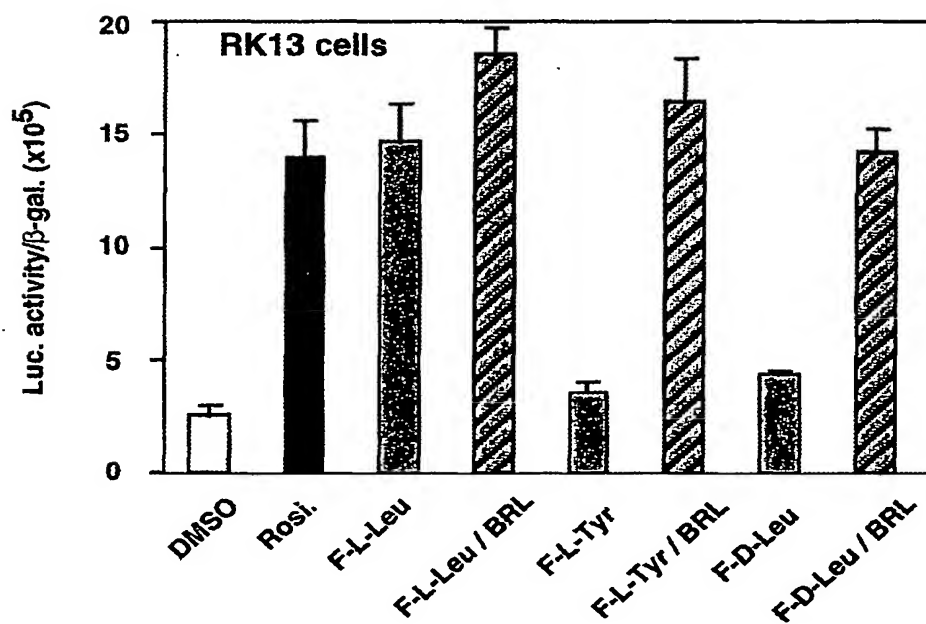


Figure 3D



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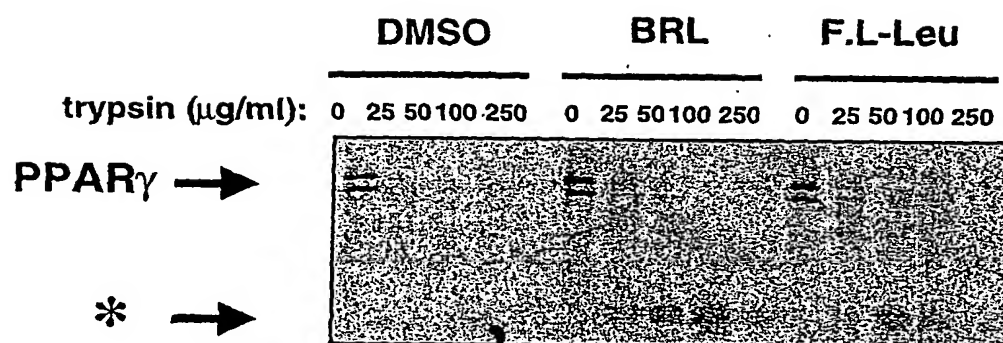


Figure 4



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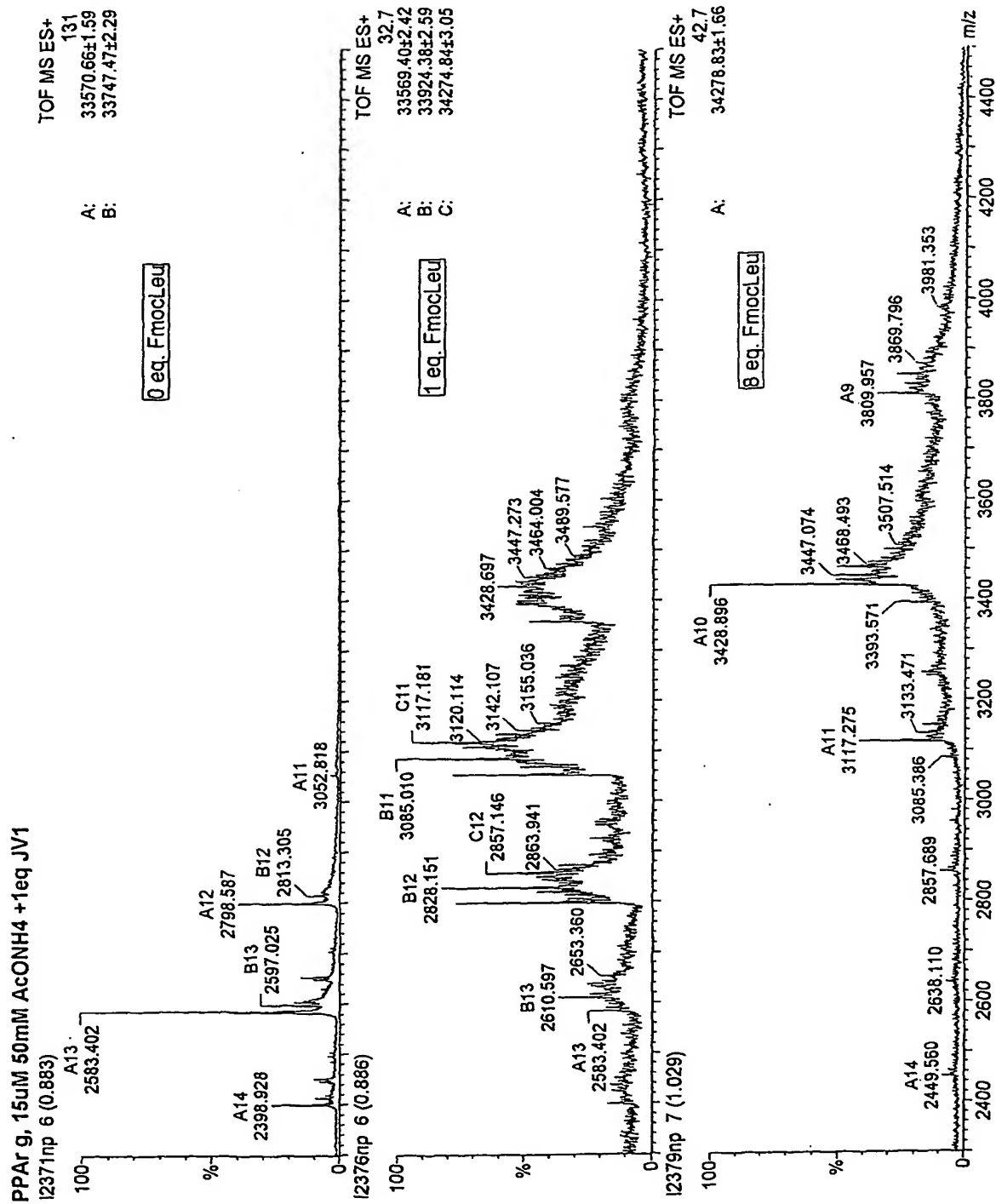


Figure 5

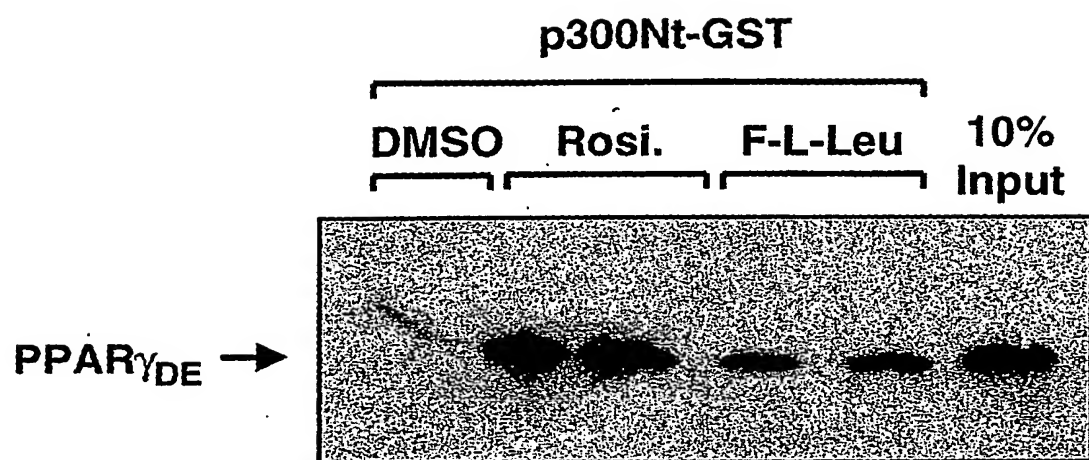


Figure 6

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Figure 7A

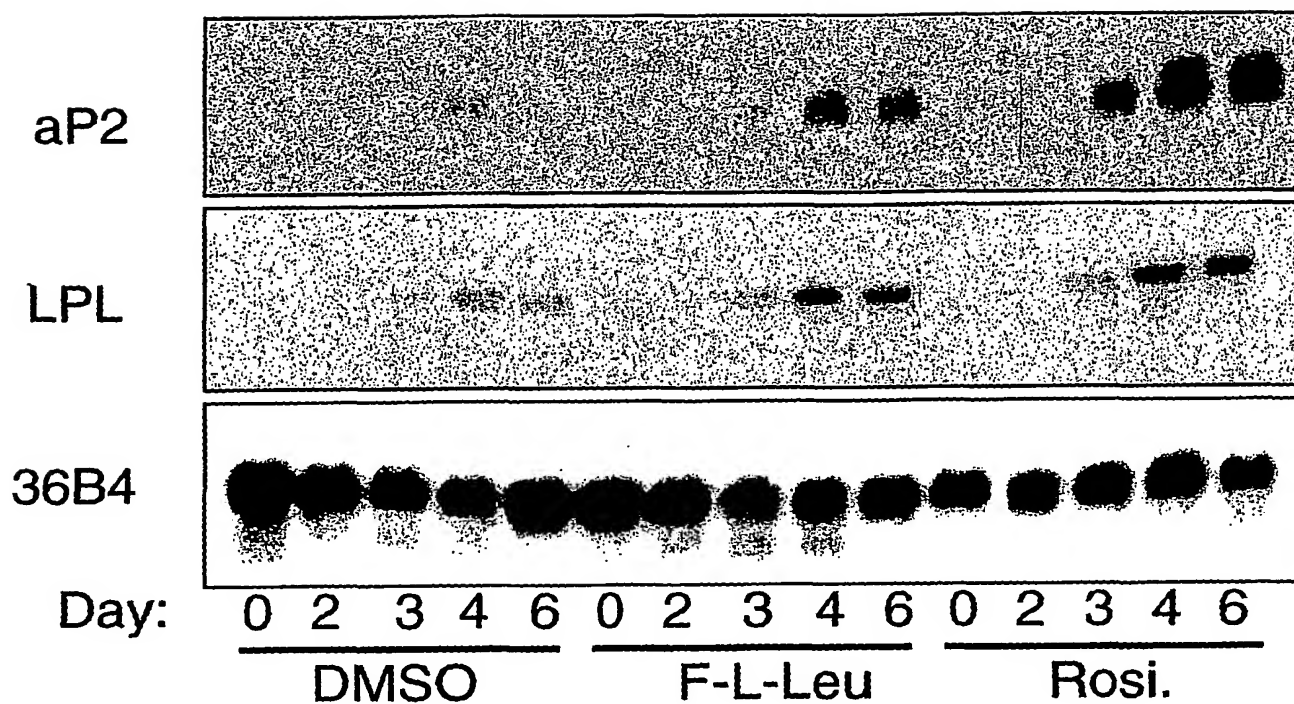
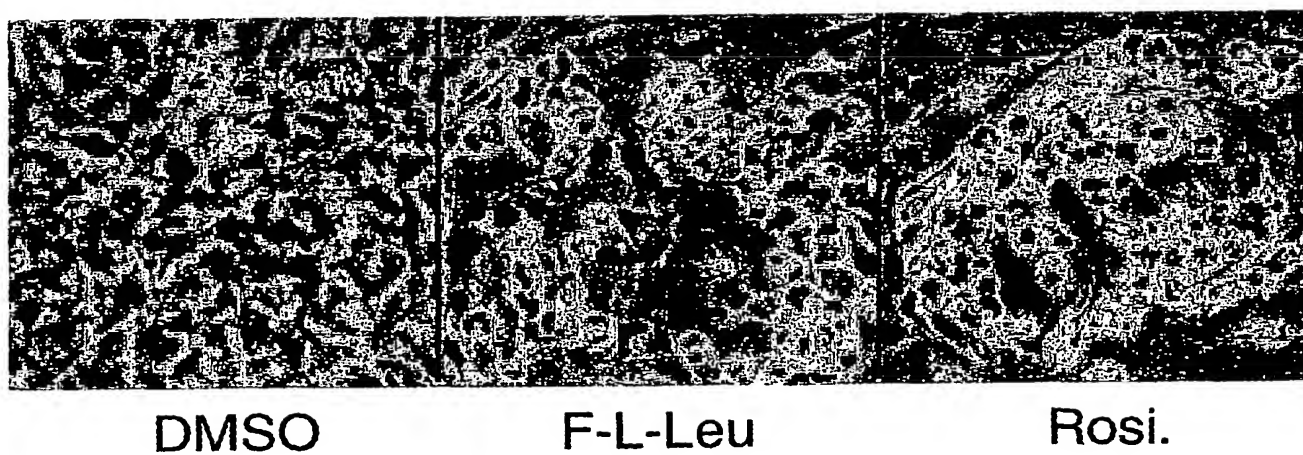


Figure 7B



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Figure 8A

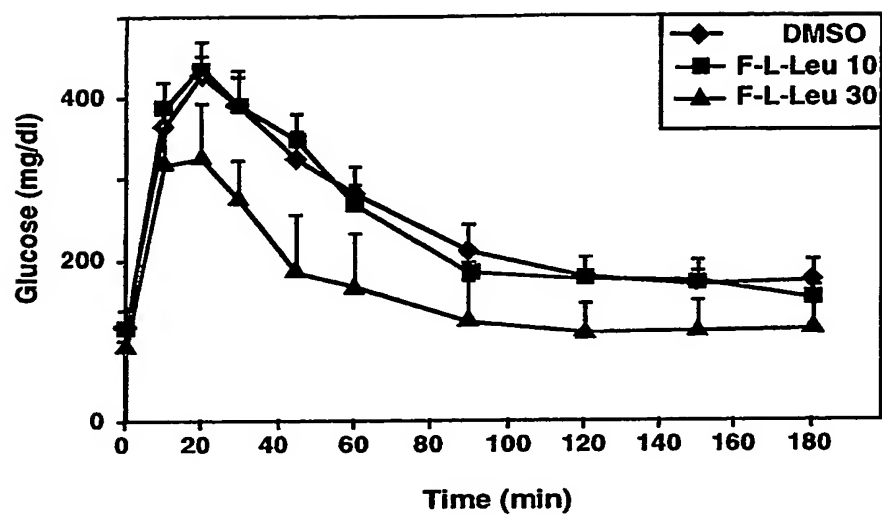
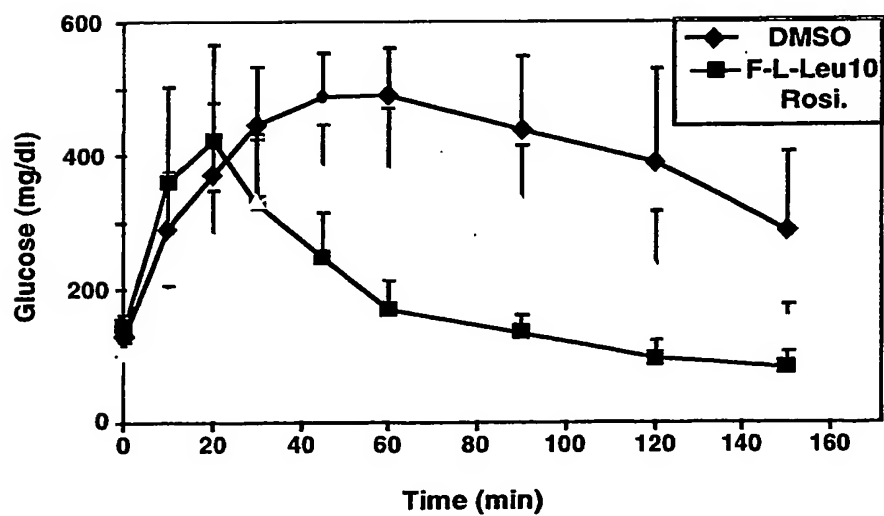


Figure 8B



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Figure 8C

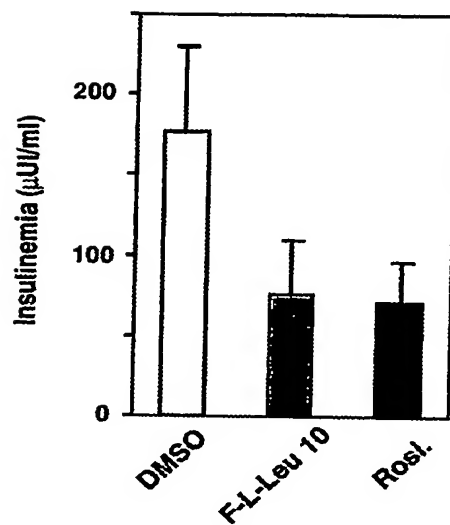
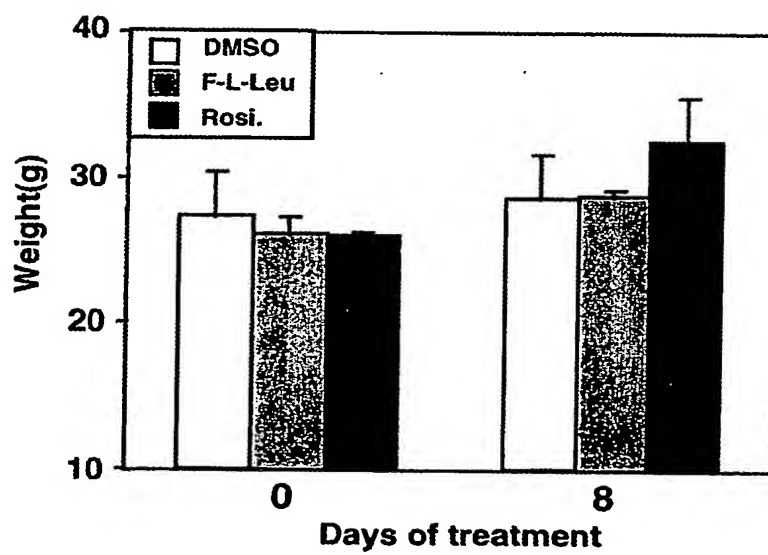


Figure 8D



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Figure 9A

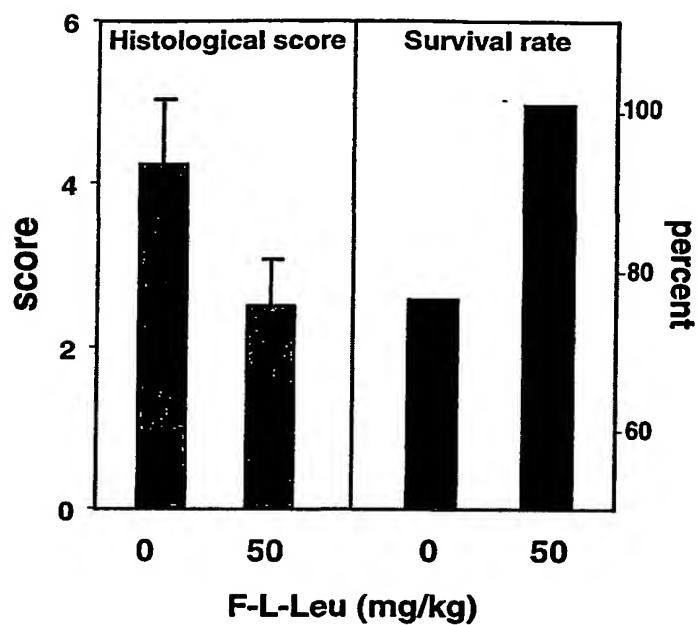


Figure 9B

